ANTIMICROBIAL ACTIVITY OF PHAGE LYTIC PROTEINS AGAINST *Staphylococcus aureus* IN DAIRY PRODUCTS

Lorena Rodríguez, Beatriz Martínez, Ana Rodríguez and Pilar García
Instituto de Productos Lácteos de Asturias, (IPLA-CSIC), Villaviciosa, Asturias, Spain

INTRODUCTION

Staphylococcus aureus **is an important pathogen associated with food poisoning and one of the main causes of mastitis in cattle. In dairy products, the presence of *S. aureus* is usually associated with contamination of raw milk from animals with subclinical mastitis, or with post-pasteurization contamination owing to improper handling of the product. Heat treatment of milk does not ensure the absence of enterotoxins produced by numerous strains since they are heat stable. One promising approach to improve and ensure the hygienic quality of these products is the use of antimicrobial proteins encoded by bacteriophages (endolysins and virion-associated peptidoglycan hydrolases). These proteins are capable of degrading peptidoglycan when applied on Gram positive bacterial cells, resulting in rapid lysis of the bacteria. In addition, its specificity offers a unique possibility for the biological control of pathogen bacteria without disrupted other microorganisms such as the microbiota or the starter cultures of fermented food. Our work aims at exploiting the antimicrobial potential of lytic phage encoded proteins as food preservatives against *S. aureus* in dairy products.**

PREVIOUS WORK

We have isolated from raw bovine milk the *S. aureus* temperate phages ΦA72 and ΦH5 and selected two lytic variants (phi-SauS-IPLA35 and phi-SauS-IPLA88). Their morphology allows the classification into the family *Shiphoviridae*.. The complete genome sequence of phages phi-SauS-IPLA35 and phi-Sau-S-IPLA88 was obtained to assess the lack of virulence traits (García et al., 2009a). They effectively inhibit *S. aureus* growth in milk (García et al., 2009b) and, curd and cheese manufacturing processes (García et al., 2007; Bueno et al., 2009).

RESULTS AND DISCUSSION

Identification of lytic activities encoded in *S. aureus* phage phi-SauS-IPLA88

In the phi-SauS-IPLA88 genome we have identified two putative peptidoglycan hydrolytic activities: the endolysin LysH5 and the peptidoglycan hydrolase HydH5 (Fig. 1A). Both proteins have two catalytic domains (Fig. 1B). We have previously characterized the endolysin LysH5 which could be added as a food preservative (Obeso et al., 2008).

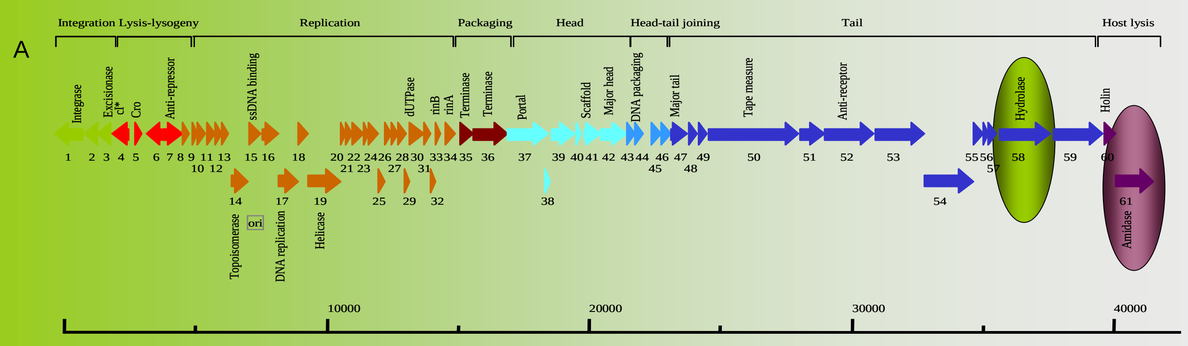
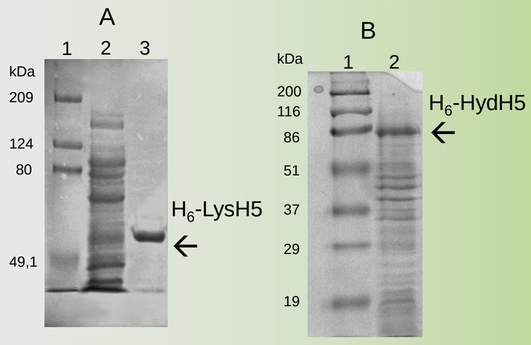


Figure 1. Identification of peptidoglycan hydrolytic activities in *S. aureus* phi-SauS-IPLA88 genome. A) Genetic map of phi-SauS-IPLA88. B) Catalitic domains of LysH5 and HydH5 proteins.

Expression of LysH5 and HydH5 in *Escherichia coli*



The endolysin LysH5 was overexproduced in *E. coli* BL21(DE3) and purified by a Ni-Agarosa affinity column (Fig. 2A). The expression of the peptidoglycan hydrolase HydH5 required the *E. coli* Rosetta DE3 strain due to the excessive rare codon usage in the target gene (Fig. 2B).

Figure 2. Expression in *E. coli* of phage-encoded peptidoglycan hydrolytic activities. A) LysH5 production and purification. B) HydH5 expression. Lanes 1: Molecular weight. Lanes 2: *E. coli* expression. Lane 3: LysH5 pure.

Heterologous production of LysH5 and HydH5 in *Lactococcus lactis*

LysH5 **(*orf61*) and *hydH5* (*orf58*) were cloned in the *L. lactis* vector pNZ8020 under the control of the nisin promoter (Pnis) (Fig. 3A). Lytic activity of both proteins against *S. aureus* cells was measured by a decrease in optical density of *S. aureus* suspensions (Fig. 3B). Specific activity (U/mg) of the *L. lactis* cell extracts was dependent of the nisin concentration (Fig. 3C).**

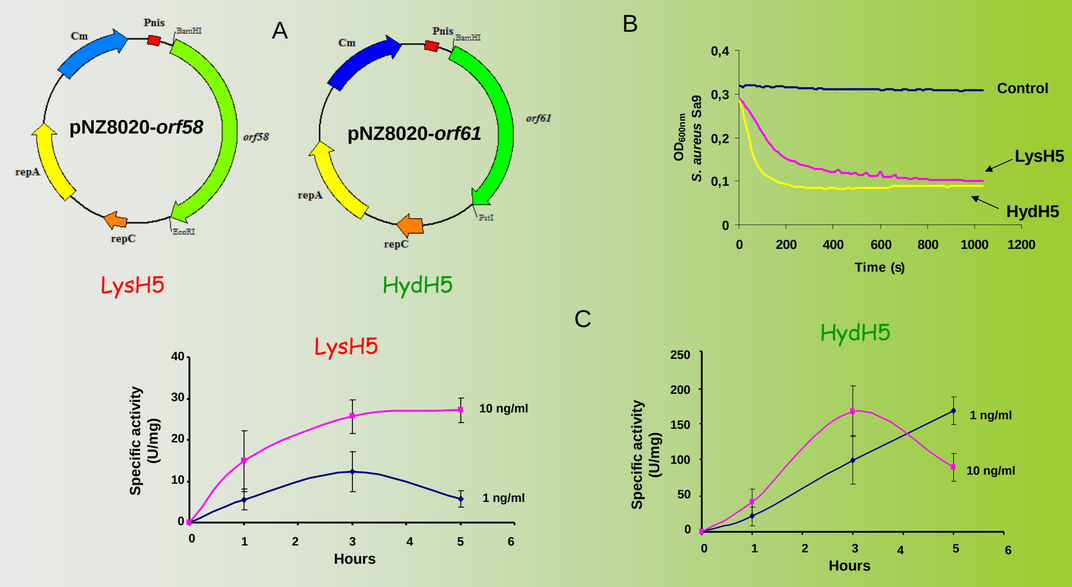
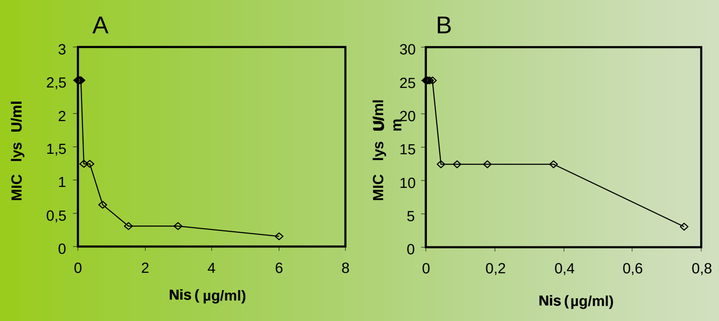


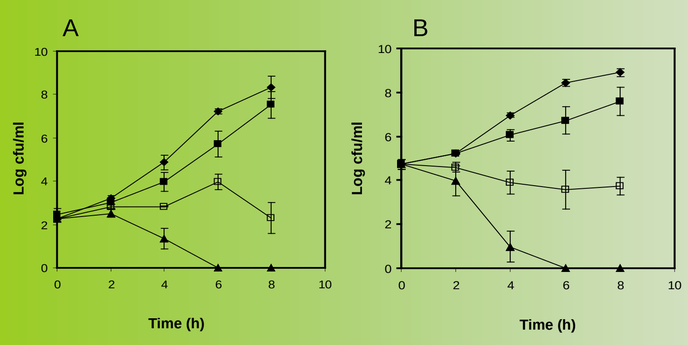
Figure 3. LysH5 and HydH5 production in *L. lactis*. A) Recombinant plasmids generated by cloning of the LysH5 and the HydH5 encoding-genes. B) OD_{600} decrease by the LysH5 and the HydH5 activity. C) Specific activity of *L. lactis* extracts containing the lytic proteins.

Synergistic effect of endolysin LysH5 and nisin *in vitro* and in milk



A synergistic effect between endolysin and the bacteriocin nisin was observed by the checkerboard microtiter test against a suspension of *S. aureus* cells in phosphate buffer (Fig. 4A) and in exponentially growing cells (Fig. 4B).

Figure 4. Changes of the endolysin MIC in the presence of nisin against A) *S. aureus* cells in buffer and B) *S. aureus* exponentially growing cells.



Antimicrobial activity of the LysH5 and nisin mixture was assesed in low (Fig. 5A) and high contaminated milk (Fig. 5B). The combination of both antimicrobial agents eliminated the pathogen bacteria in 6 h.

Figure 5. Biocontrol of *S. aureus* in milk by the endolysin LysH5 and nisin in A) low contaminated milk and B) high contaminated milk.

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